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Estrogen Receptor Isoform Gene Expression in Ovarian Stromal and Epithelial Tumors*

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ABSTRACT

The factors involved in the pathogenesis of ovarian cancers remain unclear, and the response of these tumors to hormonal therapy is limited. The identification of a second estrogen receptor gene (ERβ), expressed predominantly in ovarian granulosa cells, led us to explore its possible role in ovarian cancer, particularly in granulosa cell tumors (GCT). Several isoforms of ERβ have been identified. We sought to define the patterns of both ERα and ERβ gene expression in a panel of ovarian tumors consisting of GCT and serous and mucinous cystadenocarcinomas as well as in normal ovary. Expression was determined by RT-PCR using gene- and isoform-specific primers and probes combined with Southern blot analysis of the PCR products. Widespread expression of ERα was observed in all tumor types, but at relatively low levels. ERβ is expressed predominantly in GCT, with lower levels in mucinous tumors and very low levels in serous tumors. The ERβ2 splice variant previously reported in rodents was not observed. Only very low levels of the exon 5, exon 6, and exon 5/6 deletion variants were detected. The C-terminal truncation variant $ER\beta_{cx}$, however, exhibited widespread expression across all the tumor types. As $ER\beta_{cx}$ has been shown to be a ligand-independent antagonist of ERα action, the relative ratios of $ER\beta_{cx}$, ERα, and ERβ may influence the response of a tumor to antiestrogen therapy. (*J Clin Endocrinol Metab* **85:** 1200–1205, 2000)

ESTROGENS INFLUENCE a diverse range of functions through two estrogen receptors (ERα and ERβ) that are encoded by separate genes (1). These two receptors share both structural and functional homology. They are members of the steroid/thyroid/retinoid superfamily of ligand-dependent transcription factors (2, 3).

The granulosa cells of the developing follicle are the major site of estrogen biosynthesis in the premenopausal woman. Both estrogen actions and ERs have been identified in the normal ovary of various animal models, although some controversy exists with respect to the presence of ERs in human ovary (4). ERs and/or responses have also been reported in some ovarian tumors (5). The recent identification of the second ER (6, 7) may provide an explanation for some of the controversy surrounding ER expression in the human ovary. The major site of ERβ gene expression in the female rat (8) and human (1) is the ovary, where its expression clearly predominates over that of ERα (1, 8). Within the ovary of both species ERβ is localized to the granulosa cells (1, 6).

Several splice variants of ERβ have been identified. A 54-nucleotide insertion has been reported in the rat ERβ transcript (9), which encodes a functional receptor (10) containing 18 amino acids inserted in the ligand-binding domain (9, 10). This isoform has also been reported in mice (11) and has been termed ERβ2 (10). Low levels of ERβ expression are observed in breast tumors (12), and although the above insertion was not observed (11), shortened transcripts with deletion of exon 5, exon 6, or both have been reported (11, 13). Alternate splicing at the 3′-end of the coding region has also been reported, which results in an additional isoform, $ER\beta_{cx}$ (14, 15).

The majority of malignant ovarian tumors are epithelial in origin, but approximately 10% are classified as ovarian sex cord tumors, of which most are granulosa cell tumors (16). In this study we sought to establish which of the two ER genes is expressed in granulosa cell tumors of the ovary and to compare these with epithelial tumors. In addition, we sought to determine which of the ERβ isoforms are expressed in these tumors.

Materials and Methods

Isolation of RNA from tissue specimens

Ovarian granulosa cell tumors (GCT; n = 4), mucinous cystadenocarcinomas (n = 8), and serous cystadenocarcinomas (n = 4) were obtained from a study of serum inhibin levels in ovarian tumors (17). The tumors were consecutive tumors for which adequate tissue was available for RNA extraction. Normal ovarian tissue was obtained from three patients who had undergone elective hysterectomy with oophrectomy for menorrhagia associated with benign uterine lesions at the ages of 39, 46, and 48 yr. The clinical details of the tumors (Table 1) have been presented previously (18). The RNA was extracted using the guanidine thiocyanate/cesium chloride method as described previously (18).

RT-PCR amplification

One microgram of total RNA was reverse transcribed for 90 min at 42 C in a total volume of 20 μL using AMV reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). First strand synthesis was performed using 30 pmol of the specific antisense primer (Table 2) alone or with the β_2-microglobulin antisense primer. The oligonucleotide primers for the β_2-microglobulin gene have previously been described (18). The ER primers were designed from the published sequences (7, 19, 20) with OLIGO Primer Analysis software version 5.0

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TABLE 1. Clinical information of the patients studied

Patient no.	Age (yr)	Menopausal status	Type	Stage	CA125	Serum inhibin
1	76	Post	MC	1	53	1940
2	86	Post	MC	Benign	NA	152
3	20	Pre	MC	1	15	580
4	65	Post	MC	1 B/L	104	641
5	85	Post	MC	1	6	374
6	78	Post	MC	1	145	539
7	53	Post	MC	Unstaged	25	168
8	65	Post	MC	2	638	1090
9	46	Pre	GCT	1	11	7240
10	32	Pre	GCT	1	10	11060
11	31	Pre	GCT	1	NA	2410
12	48	Pre	GCT	Unstaged	8	112
13	56	Post	SC	Unstaged	158	1405
14	71	Post	SC	3C	412	75
15	71	Post	SC	3C	890	75
16	75	Post	SC	3C	1406	75

The inhibin normal range in postmenopausal women is below 130 U/L. In cycling women, follicular phase levels are usually below 800 U/L. The CA125 normal range is less than 35 U/L in postmenopausal women. The tumors are mucinous cystadenocarcinomas (MC), granulosa cell tumors (GCT), or serous cystadenocarcinomas (SC). In patient 12 the GCT was a recurrent tumor. One mucinous tumor is staged as borderline (B/L). Some biochemical values were not available (NA).

TABLE 2. Primer pairs and probes used for RT-PCR of the ER genes

1	Sense	5′-GCC GAC AAG GAG TTG GTA CAC AT (763)
	Antisense	5′-GGA TCA TGG CCT TGA CAC AGA GA (1072)
	Probe	5′-GATT CCC GGC TTT GTGG AGC TCA (826)
2	Sense	5′-CCG GAA TTC TTC/T GAC ATG CTC/G CTG G (981)
	Antisense	5′-GAT GC/TT CCA TGC CC/TT TGT TAC TC (1285)
	ERα Probe	5′-GTT TGT GTG CCT CAA ATC TAT TAT TT (1624)
	ERβ Probe	5′-ATA TCT CTG TGT CAA GGC CAT GA (1047)
3	Sense	5′-GGCTTC TAG ACG CCC TGA GCC CCG AGC (632)
	Antisense	5′-GGA GTT TTA ACT CTC GAA ACC TTG AAG (1036)
4	Sense	5′-GCC GAC AAG GAG TTG GTA CAC AT (763)
	Antisense	5′-TCA TAC ACT GGG ACC ACA TTT TT (1325)
5	Sense	5′-CCT GGC AAC TAC TTC AAG GTT TC (999)
	Antisense	5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G
	Probe	5′-GGA TCA TGG CCT TGA CAC AGA GA (1072)

The primer positions, numbered according to the published sequences (7, 15, 19, 20), are shown in *parentheses* at the end of each line. The sense primer in pair 3 differs at three positions at its 5′-end from the published human ERβ sequence (7) as it was originally designed from the rat ERβ sequence (9).

(Natural Biosciences, North Plymouth, MN). Primer pair 1 is for ERβ-specific amplification. The second primer pair consists of universal primers, which will amplify both ERα and ERβ. Primer pair 3 spans the exon 5-exon 6 splice junction for the detection of ERβ2. The fourth sense primer hybridizes to exon 4, and the antisense primer hybridizes to exon 8 to detect ER$\beta\Delta$5, ER$\beta\Delta$6, and ER$\beta\Delta$5/6. The primers for ERβ_{cx} are the same as those used by Moore *et al.* (15). Two microliters of each RT reaction were amplified in a single stage PCR for 30 cycles with 10 pmol gene-specific primers and 2.5 U *Taq* polymerase (Roche Molecular Biochemicals), in a total volume of 50 μL. The thermal cycling profile for the receptor consisted of a denaturing step at 95 C for 5 min and subsequently for 30 s, annealing at 60 C for 30 s, and extension at 72 C for 45 s, with a final 72 C incubation for 5 min. The products were visualized on a 1.8% agarose gel, stained with ethidium bromide, and photographed under UV transillumination. Controls for the RT-PCR were the reaction mixtures described above but with reverse transcriptase omitted. The identity of the amplicons was confirmed by direct sequencing (21).

Southern blot analysis

For Southern blot analysis using gene-specific ^{32}P-labeled probes (Table 1), the PCR products described above were transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech, Aylesbury, UK) as described previously (18, 22).

PAGE

PCR products amplified with primer pair 4 were electrophoresed through an 8% nondenaturing polyacrylamide gel and transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech) using the Mini Trans-Blot Cell Apparatus (Bio-Rad Laboratories, Inc., Regents Park, Australia). The membranes were used for Southern blot analysis as described above.

Results

Amplification by RT-PCR of total RNA from the granulosa cell tumors yielded an amplicon of the appropriate size for ERβ. The pattern of expression across the various tumors was assessed by Southern blot analysis of the RT-PCR products using a hybridization probe directed at a region within the amplification primers (Fig. 1). The pattern of relative expression for ERβ is GCT$\gg$mucinous tumors$\gg$serous tumors.

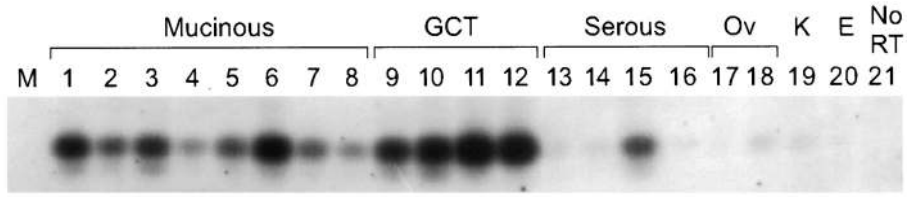

FIG. 1. Southern blot analysis of the RT-PCR products from mucinous, GCT, and serous tumors amplified with ERβ-specific primers for 25 cycles. Lanes 17 and 18, normal ovary (Ov); lane 19, kidney (K); lane 20, endometrium (E); lane 21, RT was omitted from the RT-PCR for sample 11. The first lane (M) contains the molecular weight markers. The numbers for the tumors correspond to those in Table 1.

There is, however, significant variation between tumors within each tumor type for the mucinous tumors and one of the serous tumors. This variability with the mucinous tumors does not obviously correlate with stage, age of patient, or serum hormone levels (Table 1). The relatively low levels in the normal ovary presumably reflect the relatively small number of granulosa cells in these ovaries, which are from older premenopausal women.

To explore the relative patterns of expression of ERα and ERβ in these tumors, universal primers were designed to amplify both receptors (Fig. 2). The RT-PCR also included primers for β_2-microglobulin (18) to provide an internal control. The results of the initial amplification for 30 cycles are shown in the *upper panel* of Fig. 2. To ensure that the amplification remained in the linear phase, the samples were also amplified for 25 cycles before Southern blotting with gene-specific ^{32}P-labeled internal oligonucleotide probes (Fig. 2). ERα shows widespread low levels of expression, which are similar to or less than those seen in the endometrium. The pattern of ERβ expression is described in Fig. 1. Although a direct quantitative comparison between the levels of ERα and ERβ expression is difficult, the levels of ERβ expression can be inferred from the ethidium bromide-stained amplicons in tissues that predominantly express ERβ compared with those that express only ERα. ERβ expression, at least in the GCT, is severalfold higher than that of ERα.

In our initial attempts to establish a RT-PCR assay for human ERβ to use in these studies, we identified an alternative form of rat ERβ containing a 54-base insertion in the ligand-binding domain between exons 5 and 6 (9) that was subsequently designated ERβ2 (10). To establish whether this ERβ isoform was expressed in species other than rodents and to establish whether it was expressed in a tumor-specific fashion, those tumors that clearly expressed ERβ were subjected to RT-PCR using primers that yielded an amplicon spanning the exon 5/6 junction (Fig. 3). The ERβ2 isoform was not observed in either normal ovary or ERβ-expressing ovarian tumors. The previously published results in rats (9) are also shown in Fig. 3 for comparison.

The second set of isoforms examined relate to alternate splicing of exons 5 and 6. In this study RNA from representative tumors with clear ERβ expression (Figs. 1 and 2) was amplified using primers spanning both exons 5 and 6. Although the wild-type transcript was inevitably seen, the ERβΔ5 and ERβΔ6 isoforms were extremely difficult to detect. Where faint bands were seen it was very difficult to determine which of the two isoforms they represented, given that they differed by only six nucleotides. A faint band corresponding to the ERβΔ5/Δ6 isoform was observed. Southern blot analysis of the PCR products with a ^{32}P-labeled internal oligonucleotide that would detect all isoforms is shown (Fig. 4); this emphasizes that these isoforms are an extremely minor component relative to the wild-type transcript.

Expression of the $ER\beta_{cx}$ variant (14), also termed ERβ2 by Moore *et al.* (15), was sought in the panel of tumors using a sense primer in exon 6 and an isoform-specific primer directed at the 3′-untranslated region; the amplicon thus spans exon 7. The PCR products were analyzed using Southern blot analysis (Fig. 5). Widespread expression of the $ER\beta_{cx}$ isoform was observed in all tumor types, with relative levels that showed much less variation than that observed for ERβ (Figs. 1 and 2). Also observed were two minor splice variants, ERβ4 and ERβ5, described by Moore *et al.* (15). The abundance of these forms closely parallels that of ERβcx. The identity of $ER\beta_{cx}$ was confirmed by direct sequencing of the amplicon. The $ER\beta_{cx}$ variant appears relatively abundant; however, it is difficult to make a direct comparison with wild-type ERβ; the strategy used in Fig. 2 to compare ERα and ERβ is not applicable. As the $ER\beta_{cx}$ transcript will be a component of the transcript detected by the RT-PCR used in Fig. 1 but not in Fig. 2, it would seem likely that at least in the GCT they may still make a relatively small contribution to the total pool of ERβ transcript. This may not be the case in the mucinous tumors.

Discussion

The role of the ER in the ovary has been the subject of some conjecture, particularly given their presence in the highly estrogenic milieu of the granulosa cell. In rodent models there is good evidence that estrogens synergize with FSH to stimulate granulosa cell proliferation (23). Female transgenic mice with a null mutation of both alleles of the ERβ gene have diminished fertility; their ovaries show diminished granulosa cell numbers in the follicles with impaired ovulation and corpus luteum formation (24). In the rat, ERβ is preferentially expressed in immature granulosa cells (25). The early appearance of ERβ suggests that it may be induced by FSH stimulation. FSH also stimulates aromatase gene expression in granulosa cells (26), suggesting an autocrine or perhaps paracrine feed-forward effect of estrogens on granulosa cell growth. As the LH receptor is also induced by estrogens in granulosa cells, this effect may be self-limiting as the cells luteinize (26). LH stimulation has been reported to down-regulate ERβ messenger RNA levels (27). This combination of effects may explain apparently conflicting studies in which estrogens have been reported to be both stimulating and

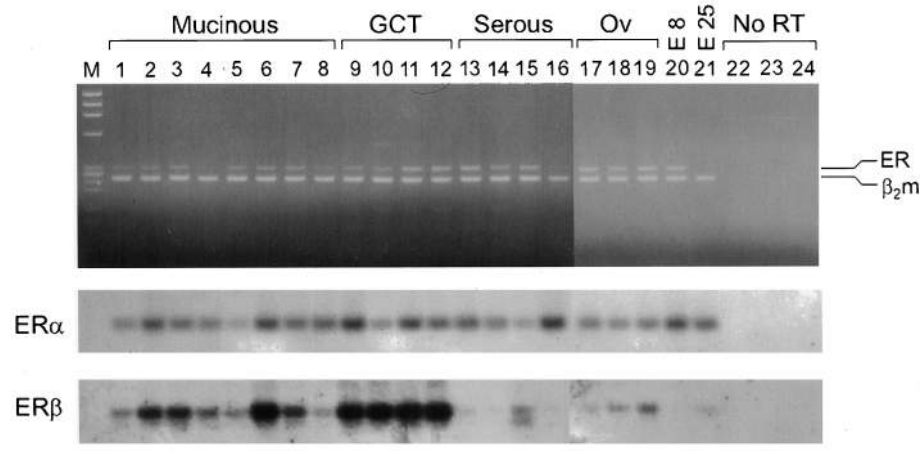

FIG. 2. Southern blot analysis (*middle* and *lower panels*) of the RT-PCR products amplified, using primer pair 2 (*upper panel*) probed with ERα- and ERβ-specific ^{32}P-labeled oligonucleotide probes. The PCR was performed for 30 cycles in the *upper panel* and 25 cycles in the *lower panels*. The tumors are described in Fig. 1. Three normal ovarian (Ov) samples are included as controls, as are samples of day 8 and day 25 endometrium (E). The RT was omitted from reactions 5, 12, and 13 in lanes 22, 23, and 24, respectively.

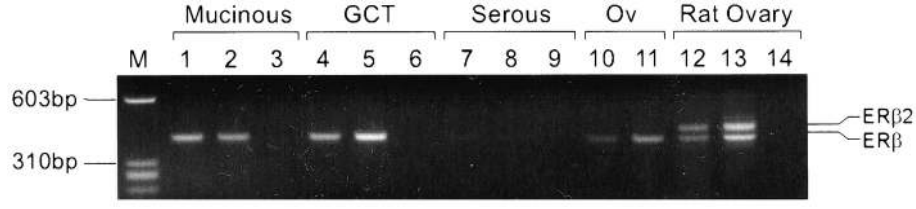

FIG. 3. Ethidium bromide-stained 2% agarose gel of an ERβ-specific RT-PCR that spans the exon 5–6 junction. Molecular weight markers (M) are shown on the *left*. Three representative tumors from each tumor type were examined (lanes 1–9) as well as RNA from two normal ovaries. The equivalent RT-PCR reaction from rat ovary (9) is shown for comparison. Samples 3, 6, 9, and 14 are the no RT controls for the preceding sample.

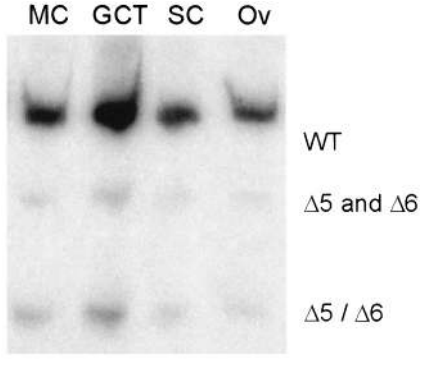

FIG. 4. Autoradiograph of a Southern blot probed with an ERβ-specific probe. Mucinous cystadenocarcinoma (MC), GCT, serous cystadenocarcinoma (SC), and normal ovary (Ov) were subjected to RT-PCR, with primers spanning from exon 4 to exon 8. The isoforms are indicated.

inhibitory. Whether the same principles apply in the human ovary is less clear (4).

Although studies of the ER in normal ovaries have focused on granulosa cells, studies of ovarian tumors have tended to focus on epithelial tumors, which are thought to arise from the simple cuboidal surface epithelium of the ovary. Hillier *et al.* (28) reported the expression of both ER genes in cultured human surface epithelial cells; whether these receptors are also expressed *in vivo* in the epithelium has yet to be determined.

The major novel findings of this study are the demonstration of abundant expression of ERβ isoforms in granulosa cell tumors of the ovary and the comparison of these expression profiles with the two subtypes of ovarian epithelial tumor. The relatively abundant expression of ERβ in the granulosa cell tumors parallels the patterns of expression for ERα and ERβ in normal granulosa cells (1). Relatively lower expression of ERβ was observed in the mucinous tumors examined, and even lower levels were observed for the serous tumors. Brandenberger *et al.* (29) reported expression of both ERα and ERβ in serous cystadenocarcinomas of the ovary, with relatively higher expression of ERα than ERβ, at least compared to that in normal ovary. Pujol *et al.* (30) explored the relative expression of ERβ and ERα in ovarian tumor cell lines and a range of benign and malignant epithelial tumors. As in the study by Brandenberger *et al.* (29), they relate increased expression of ERα to the neoplastic process. They examined eight serous carcinomas, only two mucinous carcinomas, and no GCT. Our results are consistent with both of these studies with regard to expression in serous tumors. Our larger sample size suggests, however, considerable variation in ERβ expression by the mucinous tumors, at least half of which predominantly express ERβ.

Numerous splicing variants of the human ERα gene have been reported (31) in both normal tissue and, more particularly, in human breast tumors. More recently, several splice variants of the ERβ gene have also been described, and their expression was also sought in the present study. The ERβ2 variant, a 54-nucleotide insertion at the exon 5–6 junction, has been reported by several groups in rat (9, 10, 32, 33) and murine (11) tissue, where the levels of expression of the ERβ2 isoforms appear similar to those of ERβ1 (wild-type transcript). ERβ2 is able to bind ligand and mediate expression through an estrogen response element (10, 32), but it requires

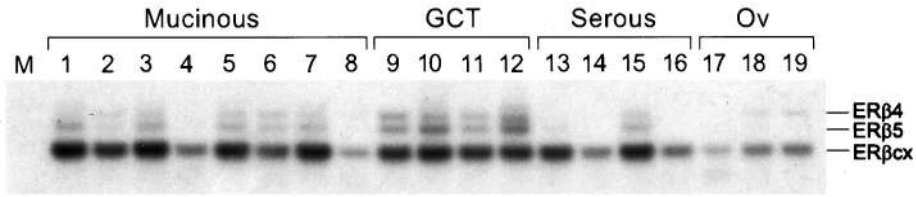

FIG. 5. Southern blot analysis of the RT-PCR products resulting from the amplification with an exon 6 primer and an ERβ_{cx} isoform-specific reverse primer. The PCR was performed for 25 cycles, and hybridization was performed with a ^{32}P-labeled oligonucleotide probe directed at sequences in exon 6 within the amplicon. The tumors and normal ovaries (Ov) are described in Figs. 1 and 2. Molecular weight markers (M) are in the first lane.

1000-fold greater concentrations of estradiol than ERβ1. ERβ2 is able to suppress ERβ1- and ERα-mediated transcription (32, 33), such that it would appear to be a negative regulator of estrogen action (33). Although its presence has been reported in a human ovarian cancer cell line (32), no evidence for its expression was found in normal ovary or ovarian tumors. Lu *et al.* (11) also failed to detect the ERβ2 isoform in human tissue. At this stage, therefore, the importance of ERβ2 appears to be restricted to rodents.

Two groups have recently reported the existence of ERβ messenger RNA variants in breast tumors (11, 13) and normal tissues, including ovary (11). Vladusic *et al.* (13) reported coexpression of wild-type ERβ and a variant in which 139 bp corresponding to exon 5 are deleted in an ERα-negative breast cancer cell line and in three of four breast tumors. Lu *et al.* (11) reported a similar finding; they examined four ERβ-positive human breast tumors and observed bands corresponding to an exon 5 deletion, an exon 6 deletion, and an exon 5–6 deletion. All are predicted to encode truncated proteins, making ligand binding unlikely (11). They reported similar transcripts in normal breast, ovary, and uterine tissue. At this stage no evidence has been presented that these variants are translated (11, 13). Our studies clearly identified coexpression of the exon 5, 6, and 5/6 deletion variants of ERβ in ovarian tumors, as reported by Lu *et al.* (11). We also found them to be of much lesser abundance compared to the wild-type transcript. The ERα exon 5 deletion splicing variant has been reported in normal ovary, uterine endometrium, and cervix and in tumors arising from these tissues (34). The significance of these variant transcripts is obscure. If they are translated into stable receptor molecules, it would be unlikely that they bind ligand; it is possible that they may act either as ligand-independent constitutively active receptors, as the ERα exon 5 splice variant does (34) or, alternatively, as ligand-independent dominant negative inhibitors of estrogen action.

Two groups have recently reported a further isoform of human ERβ that involves alternative splicing of the C-terminal region (14, 15). The predominant variant ERβ_{cx} is truncated compared with that of ERβ, but has an extra 26 amino acids at the C-terminus and a novel 3′-untranslated region as a result of alternative splicing. This pattern of splicing is also seen for isoforms of other members of the steroid hormone receptor superfamily (3). Several other isoforms resulting from alternate splicing of this region have been described, but both their abundance and tissue distribution appear relatively limited compared with those of ERβ_{cx} (15). ERβ_{cx} is unable to bind ligand (14). ERβ_{cx} preferentially forms a heterodimer with ERα rather than ERβ and elicits a dominant negative effect on ERα-mediated *trans*-activation (14). Expression of ERβ_{cx} has been reported in normal ovary (14, 15), as observed in this study, and in breast tumors (35). Ogawa *et al.* (14) were able to detect ERβ_{cx} protein in several cell lines using Western blot analysis. Most striking is the widespread expression of ERβ_{cx} in all of the tumors examined at levels above those observed in normal ovary. The levels of ERβ_{cx} expression parallel those for ERβ, although the magnitude of the variation in levels among the tumors is much less for ERβ_{cx} than for ERβ. Given that the ERβ transcripts measured in Fig. 1 include transcripts with all of the C-terminal variants, differences in the levels of expression between tumors, particularly for the GCT, must represent a predominant increase in wild-type ERβ expression. The ERβ4 and ERβ5 isoforms described by Moore *et al.* (15) were observed, albeit as a minor product whose abundance clearly closely parallels that of ERβ_{cx}.

Many of the tumors coexpress ERα and ERβ, although whether this coexpression also occurs at a cellular level has not yet been systematically examined. Pujol *et al.* (30) used *in situ* hybridization to examine ERα and ERβ expression in a serous tumor. They reported the expression of both receptors in the epithelial cells. The same may not be true of the GCT; in the normal rat ovary, ERβ expression has been reported to be restricted to the granulosa cells, whereas ERα has been reported to be expressed at very low levels (25) or to be absent (36) in these cells, but is observed elsewhere in the ovary (36). Where coexpression does occur in tumors, the observed ability of these receptors to heterodimerize *in vitro* (14) may be relevant.

The recognition that most ovarian tumors express one or both ERs suggests firstly that estrogens may have a role in pathogenesis, and secondly that appropriate agonists or antagonists may have a therapeutic role. Previous studies of the therapeutic effects of antiestrogens, particularly tamoxifen, may have been confounded by the relative heterogeneity of the tumors being examined, particularly with regard to ERα *vs.* ERβ expression as well as expression of the ERβ_{cx} isoform. Some studies suggest subtle tissue- and promoter-specific differences in the way in which ERα and ERβ respond to ligands, particularly antagonists (37–39). Consideration of the types and isoforms of ER expressed in an ovarian tumor in future clinical studies may enable the therapeutic agents to be tailored to the specific tumor.

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